Examining Efficacy of a Topical Nutrition Therapy for Endothelial Cell Tumors

Undergraduate Honors Research Thesis

The Ohio State University

College of Education and Human Ecology

Evan Schrader

January 2014

Thesis at a Glance:

Relevant Terminology:

HE	hemangioma
BBE	blueberry extract
Vehicle application	control group
EC	endothelial cell
KHE	Kaposiform hemangioendothelioma

Context:

Endothelial cell (EC) tumors are the most common soft-tissue tumors in infants and children. Their spontaneity in regional development and developmental capacity can have detrimental effects on their hosts. The EC tumors occur in 1-2% of children, worldwide (Gordillo and Sen, 2009). This occurrence is markedly higher in Caucasian children, as nearly 10% of Caucasian children will develop EC tumors compared to other ethnicities (Gordillo and Sen, 2009). Although these EC tumors are classified as benign, the body's natural immune response to the tumors can lead them to their classification as Kaposiform Hemangioendotheliomas (KHE). This KHE is an intermediate grade malignancy, due to their potential to develop Kassabach-Merritt phenomenon (Gordillo and Sen, 2009). As the immune system responds with inflammation in the tumor region and increased blood flow to the tumor, the effective response is the sequestering of blood and platelets in the tumor. Without resolution, these features then lead to anemia, heart failure, and excessive bleeding (Gordillo and Sen, 2009). KHE tumors with Kassabach-Merritt phenomenon have a mortality rate of 30% (Fernandez et al., 2009). EC tumors also embody spontaneous development in regions close to vital structures, and when paired with Kassabach-Merritt phenomenon, these tumors then become highly malignant. For example, if a tumor forms in any proximity to an infant's eye, the tumor would impair visual development, as a large red bulge of tumor flesh would slowly encroach into the infants' permanent visual field. In the same way, any tumor near the nasal cavity, the ears, mouth, or throat could cause improper development or even impede these structures entirely, causing mortality (see Fig. 1). These specific tumors in humans are called Hemangioendotheliomas which will be abbreviated as HE for the continuation.

Figure 1 Four-month-old child with a hemangioma threatening the child's airway accessibility, thus requiring aggressive treatment

The most common form of treatment for smaller, low-grade HE tumors is termed "benign neglect," as most HE tumors will resolve themselves within their lifespan of 5-9 years. However, for those patients with life-threatening tumors that interfere with vital anatomical structures (**Fig. 1**), aggressive treatment is necessary. In these cases, treatment requires potent pharmacologic agents. Physicians currently prescribe high dose steroids (Gordillo and Sen, 2009). Surgical excision is another form of treatment, however is not widely used because of the nature and scope of these tumors. Surgical excision is not a viable option when the HE tumor is in close proximity to vital structures because of potential injury to the structures themselves (Gordillo and Sen, 2009). In the cases where tumors do not threaten vital structures, though, their sheer size risk a life-threatening hemorrhage, negating the viability of surgery in most cases (Gordillo and Sen, 2009). In the few cases where surgical excision is a possibility, residual scaring and physical defects of

the invasive surgery are excessive and permanent (Gordillo and Sen, 2009). Even if the tumor is left to resolve itself via tactics of benign neglect, research shows that 50% of those children are left with a residual deformity requiring reconstructive plastic surgery (Mulliken, 1997).

Current treatment options include high dose steroids, interferon-α, and propranolol (Gordillo and Sen, 2009). These drugs not only lack a clearly defined molecular mechanism of action in the HE tumor cells, but also pose life-threatening toxicity when used over the course of several months. This is a grave danger. The list of complications associated with the use of these drugs include: femur fractures, gastric ulcers, life-threatening infections, hypotension, hypoglycemia, respiratory distress, and spastic diplegia (Gordillo and Sen, 2009).

It is necessary to develop a nutraceutical therapy that provides a non-invasive alternative to the current methods of HE tumor care. This treatment should account for a mechanism of HE tumor proliferation.

HE Tumor Model

Progress has been made in mapping the mechanism of HE tumor proliferation since Hoak et al. established and validated an endothelial cell line called Hemangioendothelioma (EOMA). EOMA can form KHE in the 129 P/3 mouse, effectively modeling the human form of this tumor. When these mice are subcutaneously injected with EOMA cells, they form KHE with 100% efficiency (Gordillo et al., 2004). Through the process of angiogenesis, the injected EOMA cells create blood-sequestering tumors via connections with the host

vasculature. These mice with KHE also develop Kassabach-Merritt phenomenon. This model has been used by many other investigators to test the effectiveness of anti-angiogenic compounds (Albini et al., 2001; Lannutti et al., 1997; O'Reilly et al., 1995; Taraboletti et al., 1995; Wang et al., 1999). With this EOMA model, significant progress has been made in mapping tumor proliferation, leading to treatment strategies.

.

Molecular Mechanisms Involving Oxidant Production

One clear component of HE tumors is severe inflammation. This inflammation is triggered by reactive oxygen species (Roy et al., 2008). As NADPH oxidase is the major source of reactive oxygen species in endothelial cells, its activity is recognized as regulating cell signaling for the observed inflammation (Roy et al., 2008), making it a logical target for mediating hemangioma formation.

NADPH oxidase is an enzyme with a catalytic core containing *gp91*. When this enzyme performs a single electron transfer, converting ingested molecular oxygen to the superoxide ion, the catalytic *gp91* component facilitates this electron transfer. In humans, *gp91* takes form in 7 different homologs, while the mouse genome contains only 6 homologs. The *nox-2* and *nox-4* homologs are of importance in both models because they are the genes found in endothelial cells. When EOMA cells were screened by PCR for all 6 homologs of *gp91*, only NOX-4 was present. With this finding, NOX-4 became the primary target in the tumor mechanism. In further progress, NOX-4 was screened against non-tumor forming transformed murine aortic endothelial (MAE) cells. Tests showed the presence of NOX-4 in quantity 69-fold greater than what was detected in the MAE cell line

(Gordillo, et al., 2010). Currently unpublished data from Dr. Gordillo's laboratory shows similar trends of high expression of NOX-4 in human hemangioma samples as well. These data are important in two ways: they verify that the mouse model accurately represents the human condition of hemangioma, and that high levels of NOX-4 expression occur in hemangioma tumors.

When NOX-4 was silenced in EOMA cells, the cells experienced a loss of function. The knockdown of NOX-4 resulted in decreased cell proliferation *in vitro* and decreased tumor size *in vivo*. The biologically active form of oxidative stress produced by NOX-4 was also revealed as hydrogen peroxide (H_2O_2), produced through the dismutation of superoxide through metabolic processes (Gordillo, et al., 2010). The final investigation with NOX-4 was live cell imaging, which lead to the discovery that hydrogen peroxide formation occurs in the nucleus of these tumor cells, indicating that NOX-4 aggregates within the nuclear membrane (Gordillo, et al., 2010).

The excess hydrogen peroxide found specifically in the nucleus makes DNA the primary target for oxidative modifications. As a relevant biomarker, oxidized DNA accumulation was found in the urine of mice harboring HE tumors (Gordillo, et al., 2010). Thus, NOX-4 activity may play a role in tumor promotion as it may serve as the main source of oxidative stress.

Global research in solid tumor growth discovered the expression of many transcription factors that are activated by oxidative stress, resulting in cell proliferation and tumor promotion. The transcription factors AP-1 (activator protein 1) and NF-kB (Nuclear factor of kappa-light-chain-enhancer of activated B cells) are redox sensitive and therefore inducible by hydrogen peroxide (Sen and Packer, 1996) derived from NOX-4.

One of the downstream targets for these transcription factors is MCP-1 (Monocyte Chemoattractant Protein 1). MCP-1 is a member of the chemokine family of proteins, a grouping of chemoattractant cytokines. This protein plays a major role in selectively recruiting macrophages and regulates the migration and infiltration of these macrophages (Deshmane 2009). Both NF-kB and AP-1 have been shown to bind to the MCP-1 promoter in endothelial cells (Martin et al., 1997).

In my work in Dr. Gordillo's lab, this is the extent of our perception of the tumor mechanism off of which this thesis is based. The findings in this mechanism provide context for the development of a nutraceutical treatment for hemangiomas, which is where my work in the research lab has honed in. Clearly, though, there are many more routes of experimentation that need to be investigated to complete the mapping of the hemangioma tumor mechanism.

A Treatment Against Tumorigenesis: Antioxidants from Berries

Since redox sensitive transcription factors are clear components of cell proliferation in the EOMA model, naturally-occurring antioxidants could be the answer to reducing the high oxidative stress found in the HE tumor. In this experiment, antioxidants will be tested via direct topical application to the tumors. Antioxidants are expected to eliminate the oxidative stress within the tumor, thereby preventing activation of transcription factors like NF-kB and AP-1. As a result, the *MCP-1* gene will remain inactive. Additionally, the elimination of reactive oxygen species in the cell nucleus will create an environment where

the cell will be able to maintain its intact DNA with greater efficiency, promoting the maintenance of healthy cells.

Berry extracts—most specifically blueberry extracts—were selected as a possibility for therapeutic intervention because their high oxygen radical absorbance capacities (Haytowitz, et al. 2010, Gordillo, 2009) enable them to quench free radicals created in the nucleus. These extracts are also rich in anthocyanins, which are antiangiogenic and can inhibit AP-1 stimulated neoplastic transformation (Roy et al., 2002). This potential antioxidant therapy appears to have properties that address significant promoters of endothelial cell tumor growth. A proprietary blend of berry extracts (PediaBerry™) will be tested in the EOMA cell model *in vitro*.

Berry extracts represent a viable therapeutic approach that could be available to all children with endothelial cell tumors. It is critical to study the topical application of berry extracts in reducing the oxidative stress present in HE tumor cells in order to create a nutraceutical and non-invasive therapy option for patients with HE tumors.

Relevant Biomarkers in Tumor Proliferation

Besides techniques in recording lifespan rates and volumetric measurement of the tumors, various protein biomarkers exist in relation to hemangioma research and are important to this study. Collagen-1 is a fibrous protein that supports tissue structure, among many other roles. Collagen-1 is a protein chosen as a relevant biomarker of tumor proliferation in this study because of its previously established role in tumor research. One study found that the presence of Collagen-1 enhanced tumor cell proliferation (Ki 2014). In another study, Collagen-1 was found to upregulate the expression of MT1-MMP, a protein

which induces the metastasis of disease (Ki 2014). Adding to this, research has also shown that regions of tissue adjacent to benign tumors reveal high levels of collagen-1 deposition. Research has linked this finding to a greater flux in macromolecule transport to tumor cells (Kakkad 2013), increasing tumor proliferation. When antifibrotic drugs successfully treated leiomyomas, benign smoothe muscle neoplasms, this research indicated that Collagen-1 plays a role in tumor maintenance as well (Islam 2014). Using these cases, Collagen-1 serves as a biomarker fit for determining the effects of PediaBerry™ on HE tumor proliferation.

Ki-67 is another biomarker that can provide clear, significant insight in the cell proliferation taking place in this study. Expression of Ki-67 protein is associated directly with cell proliferation (Scholzen 2000). During all active phases of the cell cycle Ki-67 protein is present, yet is absent from resting cells (Scholzen 2000). The expression of Ki-67 makes it an excellent maker for determining cell proliferation. Scholzen provides numerous references to reputable studies that have used Ki-67 as a cell proliferation protein biomarker. The relevance of Ki-67 to other studies was carried over into this study.

F4/80 is the final biomarker relevant to this study. Reverting back to the mechanism which discovered that MCP-1 is a downstream target of the redox-induced transcription factors, MCP-1 is known to be responsible for recruiting macrophages to sites of disease and inflammation, facilitating angiogenesis (Atalay et. al 2003). This finding in macrophage activity in tumor sites provides another relevant biomarker into the angiogenic processes in hemangioma. F4/80 is a well-characterized and extensively referenced macrophage marker (Austyn and Gordon 1981). In addition to recognizing kupffer cells, Langerhans cells, peritoneal macrophages, and various others, F4/80 stains

microglia as well (Lawson et al. 1990). Research has shown significant adhesive properties of the anti-F4/80 antibody in connective tissue, which provides support for its implementation in this experiment.

Research Question:

Does the topical application of PediaBerry™ suppress hemangioma tumor growth and promote longer lifespans of mice with hemangioma tumors?

Hypothesis:

My hypothesis was that daily, topical application of PediaBerry™ ointment to induced murine HE tumors would significantly reduce the size of tumors and significantly increase the lifespans of the mice affected with the tumor.

Methods:

Animals:

129 P/3 female mice (n = 15) at 4 weeks of age were obtained from Jackson Laboratories and allowed 7 days of acclimation prior to treatment. Mice were housed in The Dorothy M. Davis Heart and Lung Research Institute, an Association for Assessment and Accreditation of Laboratory Animal Care International approved animal facility. They were kept 4 per plastic hanging cage (45 × 24 × 20 cm). Colony rooms were maintained at 23 ± 2°C with 50 ± 10% humidity on a photoperiod of 12L:12D (0600 h–1800 h). Food (Purina Rodent Chow #5001) and water were provided by the Institute. Tap water (Columbus, OH water) was centrally located in each cage. All animal procedures have

already been approved by the Institutional Animal Care and Use Committee of the National Health and Environmental Effects Research Laboratory of the U.S. Environmental Protection Agency (U.S. EPA) for the research occurring in Dr. Gayle Gordillo's laboratory in DHLRI.

Hemangioma Tumor Induction:

From cell culture, 20 T75 plates of EOMA cells were collected. Once washed with Phosphate Buffered Saline (PBS), they were centrifuged into a pellet. HBSS was used because it is a cell signaling buffer used to minimize cell death during cell transfer procedures. The pellet was diluted 1:20. To count the cells, Trypan Blue Stain (0.4%) was used. Based on the number of cells in solution, a master solution of cells was created with a concentration of 5 million cells per 100 uL of PBS. 20 syringes were then prepared for injection, each with 100 uL containing PBS and 5 million EOMA cells. Each mouse was sedated under local anesthetic. The posterior of the mouse was shaved via electric trimmer and the skin prepared with Nair. The skin was washed with water and thoroughly dried. Cells were injected beneath a small patch of skin on the posterior of each mouse. Within 3-4 days, the EOMA cells had connected with the vasculature of the host mouse and the tumor was visible by the naked eye.

PediaBerry™ Application:

Each morning at 11am the mice were treated with PediaBerry™ ointment at the surface of the tumor. The tumor was then covered with a thin adhesive Tegaderm film to ensure the treatment was not removed after application. Every three days, the skin surface was shaved, prepared with Nair, and washed with water before application to ensure full

contact and absorption of the ointment. The mice will not be sedated after the initial injection day, however, because receiving full anesthetics each day is unnatural and could become a confounding variable. Eight mice were treated daily with PediaBerry™ while seven received a vehicle application (control group). The vehicle application was an application of all components except for the berry extracts, meaning that the control group received the cream in which the extracts were suspended, as well as the Tegaderm film. This experiment was capped at 10 days because of data from previous longevity studies showing that mice with untreated hemangioma tumors do not survive much past 10 days after injection (Gordillo, 2009).

An additional longevity experiment ran in a separate set of mice. Each morning, headcounts were taken in both treatment and control groups, creating a recording of each mouse's lifespan.

Tumor Analysis:

General tumor size after 10 days of treatment was compared with the starting tumor size on Day 3 (when tumors established a connection with the host and application began), and the same was done to the tumors of the control group. After collecting the tumors, success of treatment was measured via protein biomarkers. Proteins were tagged and processed via fluorescent imaging and included Ki-67, a cell proliferation biomarker, Collagen-1, which indicates tumor rigidity, and F4/80, a macrophage biomarker. DAPI was used as a standard cell nuclei indicator.

Results:

Survival rates for each group of mice were determined based on the headcounts taken each morning. As seen in **Figure 2**, the mice treated with PediaBerry™ survived significantly longer than those in the control group. At the point in time when 100% (n=7) of the control group had expired, 50% (n=4) of the treatment group was still alive. Some mice in the treatment group (n=2) lived over 2 times longer than the longest living mice in the control group (n=3). At the point when all of the mice in the control group had expired (n=7), 75% (n=6) of the mice from the treatment group were still alive.

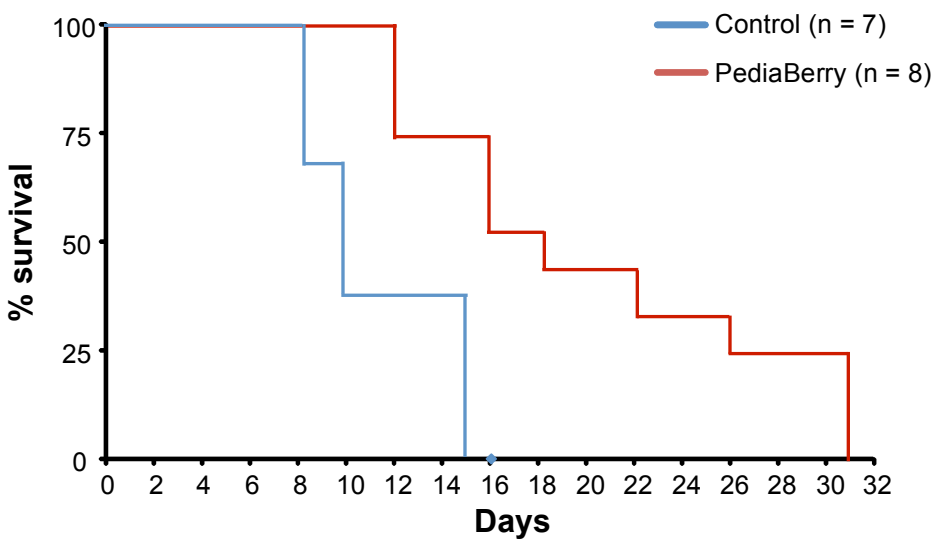
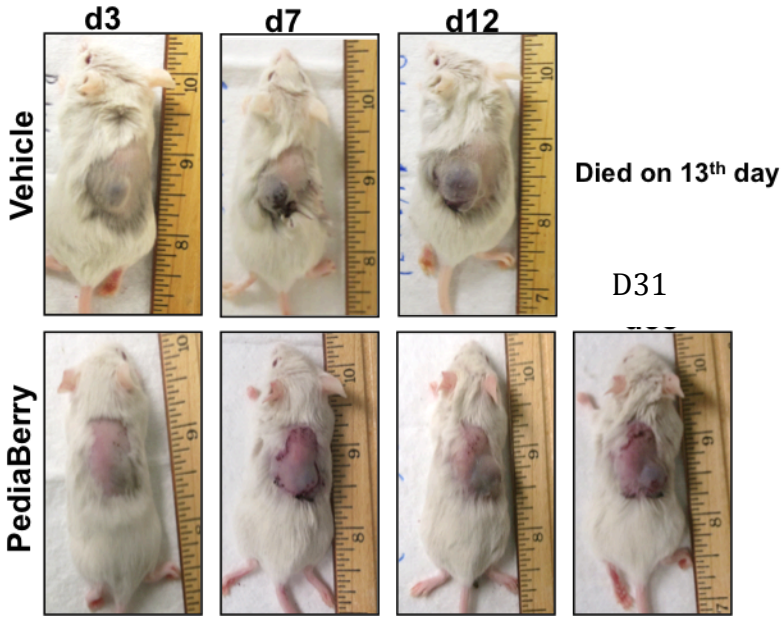


Figure 2: Increased survival rates of mice treated with PediaBerry™

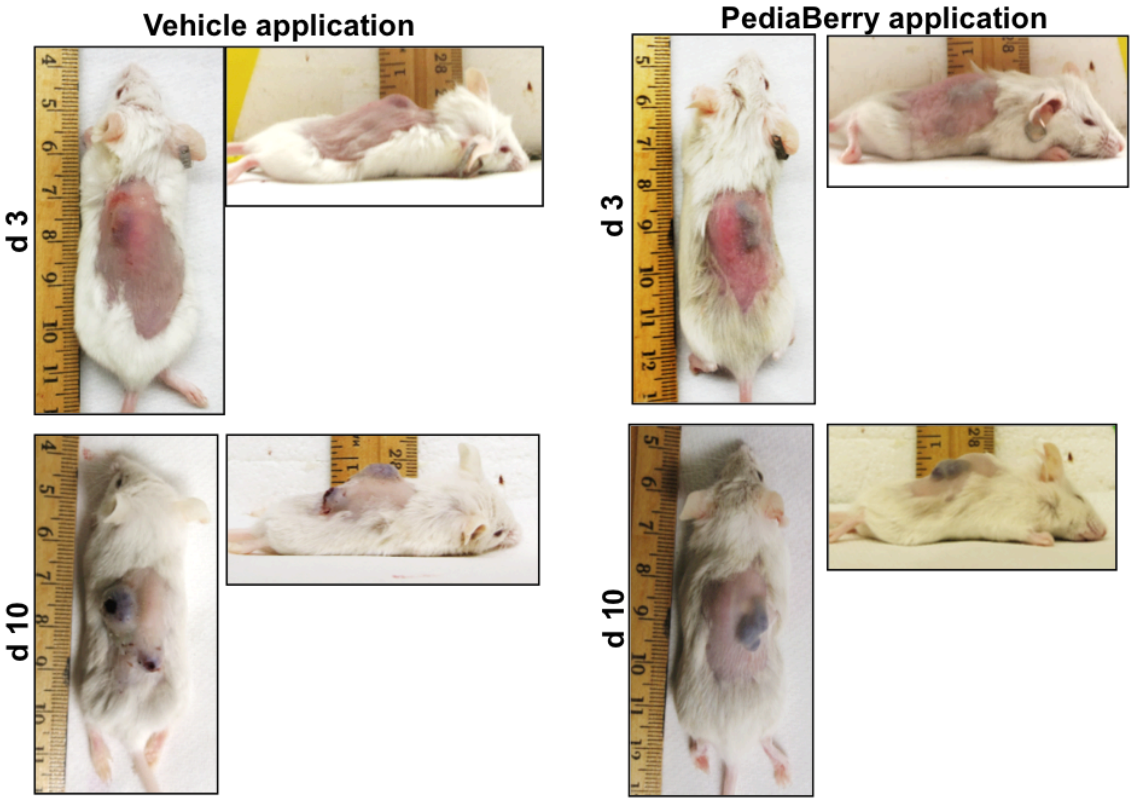
Figure 3: Mice treated with PediaBerry™ have smaller tumors and longer lifespans (mice not shown are accounted for in Figure 5)



In this longevity study, pictures were captured of the mice as a visual display of macroscopic tumor display. As shown in **Figure 3**, tumor size was recorded on Day 3, the beginning of the experiment, and on Days 7 and 12. For comparison, the mouse surviving the longest in the vehicle group is shown against a mouse whose treatment prolonged lifespan just short of three times that of the control group. The mouse in the vehicle group expired on Day 13 while the mouse treated with PediaBerry™ expired on Day 31.

The second study performed in vitro was a comparative study where the mice in both groups were sacrificed at Day 10. Captured here at Day 3 and Day 10, **Figure 4** shows the differences in tumor size from the first to the last day of study. The tumor treated with PediaBerry™ did not increase in size and its size was rather well maintained. The tumor withheld from treatment formed two tumors which both grew significantly larger than the original tumor.

Figure 4: Initial and final tumor size comparisons show maintained tumors in PediaBerry™ group (mice not photographed quantify the trend in this figure in Figure 5)



Each tumor was excised from the mouse and measured volumetrically. As shown in **Figure 5**, mice in the treatment group (n=8) had significantly smaller tumors than mice whose tumors were left untreated (n=7).

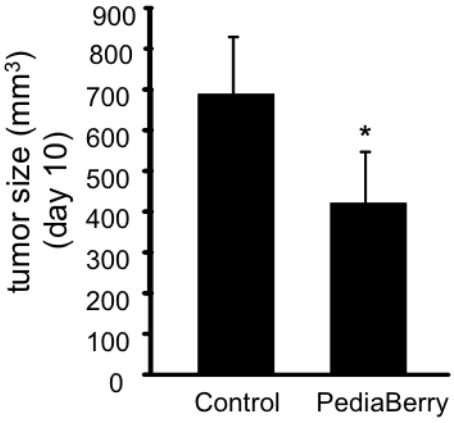
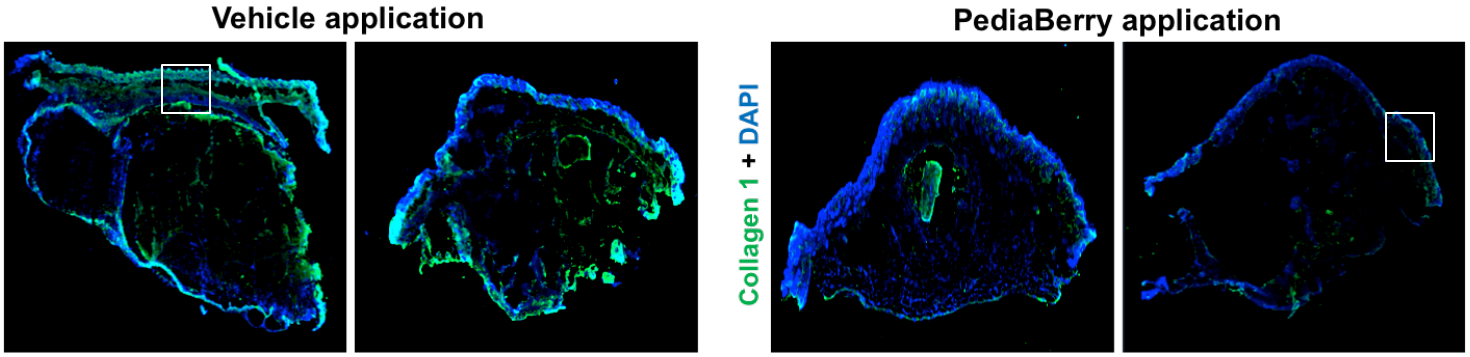


Figure 5: Decreased tumor size in mice treated with PediaBerry™

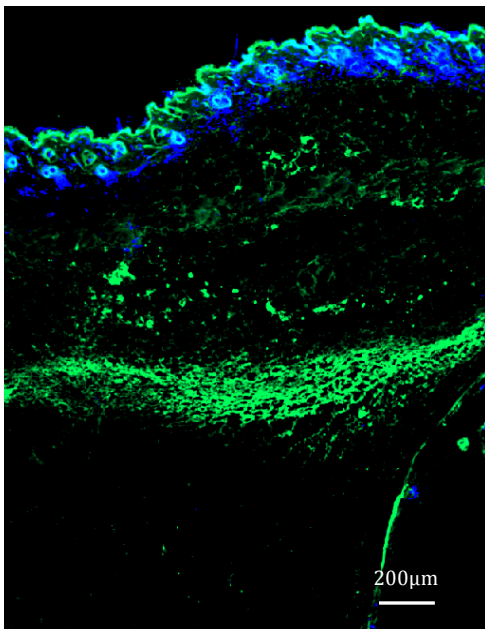
The tumors were further analyzed via fluorescent-tag protein analysis. All tumors were cut to include the entire tumor as well as the section of the mouse's tissue between the host organism and the tumor. With this methodology, the tumor sections enabled the determination of not only the content of the tumor but the transition from the mouse's connective tissue to the tumor as well. The stains that were used were for Collagen-1, F4/80, and Ki-67.

Cryosections of the vehicle application and treatment applications were stained for Collagen-1 protein content, which is indicated by fluorescent green. **Figure 6** shows that the vehicle group had much higher levels of collagen content than the group treated with PediaBerry™.

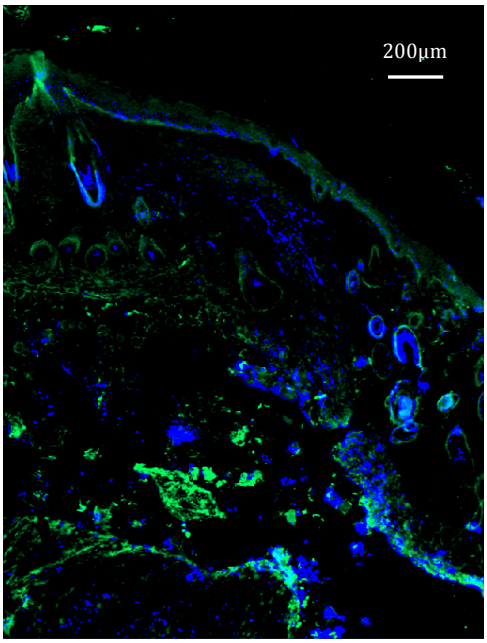
Figure 6: Decrease in Collagen-1 content shown by green fluorescence in cryosections treated with PediaBerry™ with magnified view below



Vehicle Application



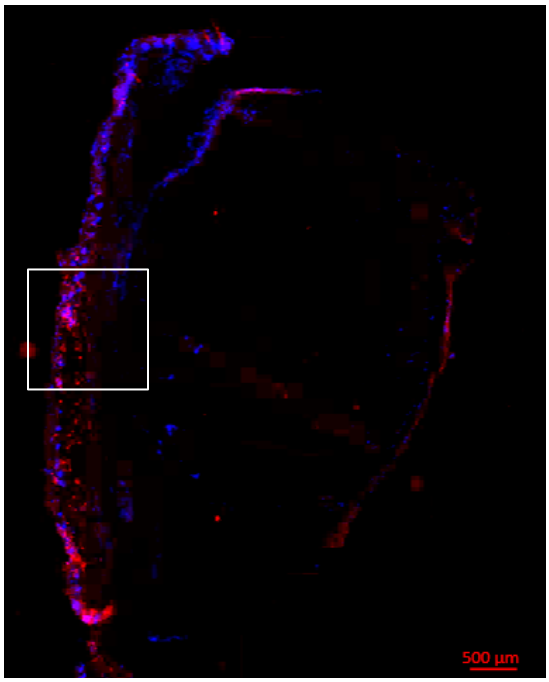
Treatment Application

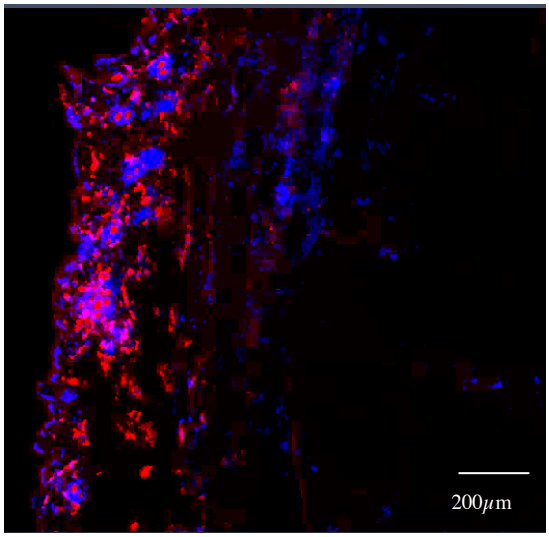


Tumors were also stained to show the protein F4/80, a biomarker for macrophages. In **Figure 7**, macrophage content is compared between tumor sections of the vehicle and treatment application. Macrophage content appears similar in both groups.

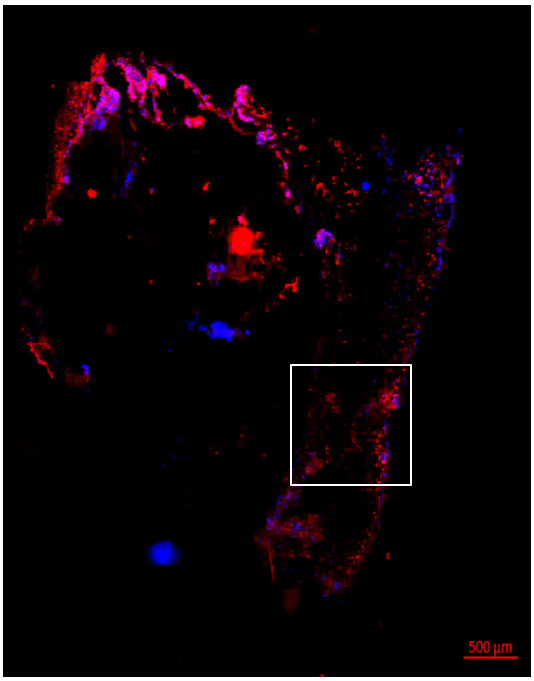
Figure 7: Similar F4/80 protein content shown with red fluorescence in tumor cryosections with magnified views outlined and shown beneath.

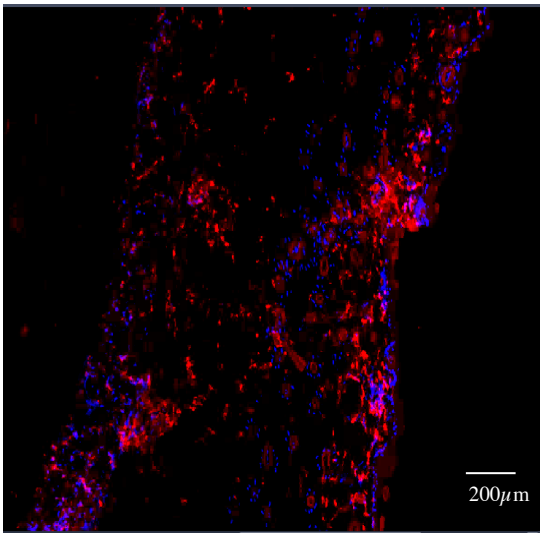
Vehicle Application





Treatment Application

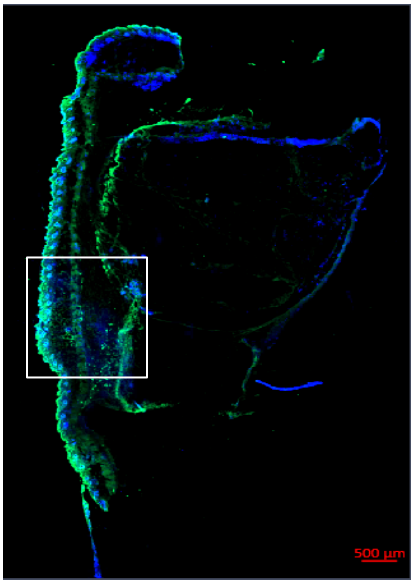


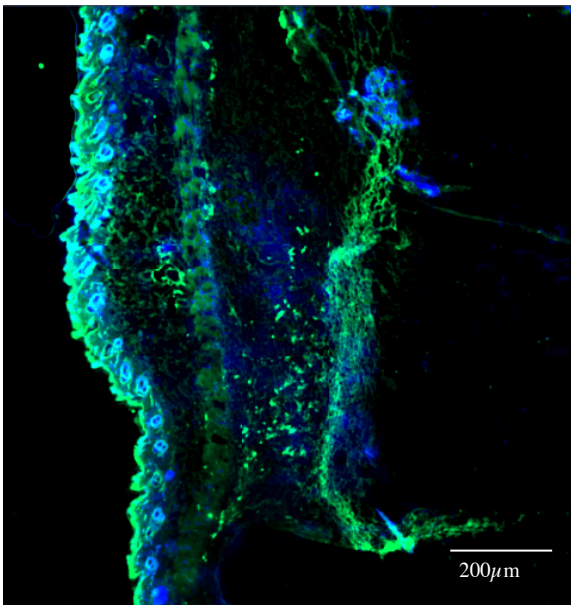


Tumor sections were stained for Ki-67, a cell proliferation protein marker. **Figure 8** shows that in the control section, cell proliferation seems to take place only in the mouse's connective tissue. The fluorescence in the control group is clear and bright. In the treatment group shown below, however, any significant Ki-67 concentration was eliminated.

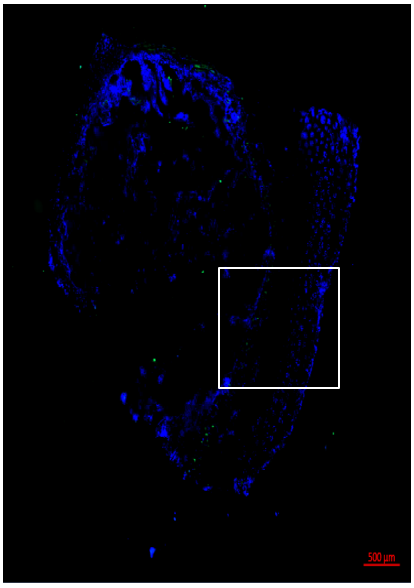
Figure 8: Significant decrease in Ki-67 content shown in green fluorescence in the cryosections with magnified views outlined and shown below

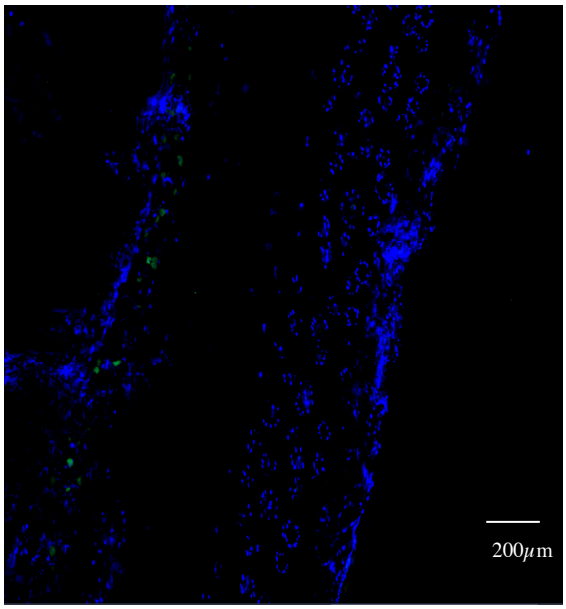
Vehicle Application





Treatment Application





Analysis:

The results of this study provide promising evidence and routes into further study. Through the longevity study on survival rates, the PediaBerry™ ointment shows evidence of tumor suppression and prolonged lifespans. The experimentation on tumor biochemistry provides further evidence of the success of PediaBerry™ ointment on a molecular level.

In the first study conducted, mice were treated with PediaBerry™ ointment and tested until expiration. As seen in Figure 2, the mice treated with PediaBerry™ ointment lived significantly longer than those untreated. Figure 3 depicts the visual differences in tumors between those treated with PediaBerry™ and those left untreated. In this figure, the control mouse saw significant tumor growth in 12 days, followed by its death. The mouse treated with PediaBerry™, however, maintained the same tumor size from Day 3 until Day 31. This data is promising evidence of the success of the ointment since the tumors were the same size at the beginning of the experiment and saw completely different paths, due to PediaBerry™. From this in vitro study, the ointment proves to work well in suppressing tumor growth and promoting survival of the organisms inflicted with this tumor. Additional support for the beneficial effects of PediaBerry™ ointment was quantified in Figure 5. This example shows the differences in overall tumor volume between the two groups. Clearly, tumors were smaller after PediaBerry™ treatment. Since Figure 5 provides results on all of the tumors of each group, this depiction also supports the validity of the single mice chosen as representative mice in Figures 3 and 4. The smaller tumors provide clear evidence to any observer that PediaBerry™ works in reducing tumor size and proliferation.

Although PediaBerry™ shows clear evidence on a macroscopic level, testing was done on the molecular level in order to gain a better understanding of the effects of PediaBerry™ application. The two most promising stains were for Collagen-1 and Ki-67. Collagen-1, the fibrous protein found in rigid structures, is much higher in the tumors omitted from PediaBerry™ treatment. As Figure 6 demonstrates, there is a drop in Collagen-1 content in the tumor sections of the treated tumors. These results support the data displayed in Figures 3 and 4 where smaller tumors were reported. Macroscopically, the tumors withheld from treatment grew in size, which explains the greater Collagen-1 content in the vehicle application tumor sections in Figure 6. Lower Collagen-1 levels in tumors treated with PediaBerry™ provides molecular data to support the smaller tumors seen in Figures 3 and 4, moving towards the idea that the ointment plays a role in collagen content. These data provide context for an extension of this experiment in determining if PediaBerry™ inhibits collagen deposition or if it degrades existing collagen, or perhaps both . Without collagen deposition, the tumor is unable to form a rigid tissue structure necessary for further growth. With collagen degradation, the tumor is unable to maintain a rigid structure, which would also inhibit proliferation. This stain on Collagen-1 demonstrates the positive effects of PediaBerry™ on a molecular level.

Ki-67 protein stains show even greater results for the success of PediaBerry™. In Figure 8, the treated tumors show significant decreases in the cell proliferation protein biomarker compared to the sections of untreated tumors. In the control group, a significant amount of Ki-67 is found throughout the tumor section. This Ki-67 content, however, is almost eliminated entirely when the tumor is treated with PediaBerry™. Low levels of Ki-67 in the tumors treated with PediaBerry™ show that cell proliferation

decreases in the tumor treated with PediaBerry™. This molecular data is seen directly in Figure 3 as the treated tumor's growth and expansion is stunted greatly compared to the tumor left untreated. This molecular data provides strong support for the viability of PediaBerry™.

The same tumor sections were stained for F4/80, a macrophage biomarker, to visualize the activity of the immune system in combatting the tumor. The images in Figure 7 show no significant differences in the macrophage content between the treated tumors and those in the control group. However, the detail of the macrophage stain is important information left undiscovered by the F4/80 stain. The origin (mouse or tumor), type, and exact roles of the macrophages are areas left uncharted by this work that would provide significant insight into the resulting macrophage content seen in Figure 7.

The Next Steps:

The macrophage stains open the doors to a great expansion of the PediaBerry™ treatment project. As seen in Figure 7, macrophage content remained constant with or without treatment via PediaBerry™. As the groups receiving PediaBerry™ treatment had much greater survival rates (Figure 2), this prolonged lifespan could be attributed to undiscovered macrophage activity. With clear macrophage aggregation in both application groups, the mystery behind the type and role of the macrophages leads to the next viable approach for this experiment. Macrophages maintain the ability to transform between two types of phenotypes, which possess counterbalancing functions in tissues. M1 phenotype macrophages function in the standard manner—they are activated in response to bacteria or xenobiotic materials and promote inflammation (Mills 2012). A second phenotype of

macrophages exists, however, which acts by changing phenotypes from a proinflammatory mediator profile to an immunosuppressive one. This M2 phenotype has been shown to protect cells from apoptosis. M2 macrophages act by withdrawing cytokine in macrophages that have phagocytized apoptotic cells (Weigert 2006). The phenotypic expression of M1/M2 down regulate each other, demonstrating the importance of adaptive immunity in a counterbalanced system (Mills 2012). M2 macrophage activity has been reported directly in endothelial cells (Reddy 2002, Golpon 2004), providing viability to the extension of this endothelial macrophage investigation. Applying this knowledge on macrophage phenotypic differentiation, further investigation can reveal the type of macrophage present in the F4/80 stains of this study. Theorizing towards further projects, tumors could be injected with manipulated phagocytic target materials, such as with protein marker tabs, which will determine the phenotype of the macrophages. With the ability to manipulate or even harness macrophage activity, further experimentation could provide means for pharmacological control of immune response to disease, specifically the inflammatory response to the endothelial cell tumor Hemangioma.

After finding a decrease in collagen in the tumors treated with PediaBerry™, further experimentation in this route could include focus on fibroblast content. Fibroblasts, being the cells that create and lay down collagen matrices, could lead to a further explanation into how PediaBerry™ works on the tumors. The fibroblast specific protein 1 (FSP-1) is a biomarker that could offer insight into the content and location of fibroblasts in the tumors (Strutz et al. 1995). Ultimately, there is either a significant fibroblast population in the HE tumor cells, or an overactive fibroblast growth factor (FGF) present in the tumor cells which would lead to the deposition of collagen seen in this study. Some FGFs are potent

angiogenic factors and can play a role in tumor growth and angiogenesis (Powers 200). Research may be able to determine how PediaBerry™ alters fibroblast and FGF activity. This data could then explain the collagen decrease seen in this study. Fibroblast activity may be altered by PediaBerry™ application, or FGF activity may be altered significantly. PediaBerry™ application may also support the degradation and relocation of collagen in the tumors. Overall, an investigation in the realm of fibroblast-related activity is important in explaining the results found in Figure 6. How and why is the collagen content decreased? The research gap in the PediaBerry™ study regarding fibroblast activity is an important opportunity to build upon the collagen findings of this study.

The final extension of the PediaBerry™ project grows off of the antioxidant properties in the PediaBerry™ ointment itself. The antioxidant properties in PediaBerry™ ointment are found within the anthocyanins of the blueberries. In the PediaBerry™ ointment used in this study, however, the antioxidant capacity is unknown, as the level of active anthocyanins was never measured. Anthocyanins are known to alleviate oxidative stress processes—the entire reason for berry utilization in the first place—placing a great importance on the simple measurement of anthocyanin levels in the ointment. An extension to this study would be a repetition study that compares different levels of anthocyanins in the PediaBerry™ ointment. Additionally, anthocyanins have been found to have cytoprotective functions in downregulating inflammatory cytokines and suppressing cellular signaling pathways of inflammatory processes (Sodagari 2015). This study on anthocyanin activity provides full-circle support for the macrophage phenotype investigation. Specifically, this study provides support for a hypothesis that PediaBerry™ ointment invokes the M2 phenotype in macrophage content as it downregulates cytokines.

As anthocyanins present significant areas for further research in antioxidant properties, flavonoids are also another compound present in berries, known to have similar redox effects (Bagachi 2006, Roy 2002). The impact of flavonoids are another mystery in berry extract research left unresolved. This extensive study done on PediaBerry™ ointment provides the basis for multiple experiments to add onto developing an effective nutraceutical to combat the proliferation of hemangioma tumors.

Significance:

Nutritional interventions for HE tumors are particularly appealing given the young age of the affected population and the absence of non-invasive treatment options. Berry extracts represent a viable therapeutic approach. The successful execution of this project provides evidence of significantly smaller tumors and longer lifespans via PediaBerry™ treatment. This study provides the link between a micronutrient found in berries and their ability to treat HE tumor pathology. This information is a key step in the development of a non-invasive treatment option for HE tumors to prevent debilitating consequences of HE tumors and ultimately save lives. The success of this in vitro study has seen the final stages of animal trials and since has provided evidence of success. PediaBerry™ ointment will soon be ready for clinical trials. Further research could produce a pharmacological agent that will provide non-invasive nutraceutical therapy to individuals suffering from Hemangioma tumors all around the world.

Acknowledgements:

I would like to thank The Ohio State University Wexner Medical Center, The Ohio State University College of Medicine, and The Ohio State University College of Education and Human Ecology for their collaboration with this Honors Research Thesis. I would like to thank Dr. Gayle Gordillo for the introduction to her research ideas and goals, her guidance through the process, and for the opportunity to learn and discover in her research lab. I would like to thank Dr. Ayan Biswas for the support and guidance in everyday research protocol and safe practices. I would like to thank him for his patience through my research and his endless help in understanding molecular practices, theory, and research direction.

I would like to thank Dr. Ouliana Ziouzenkova for all of her assistance in creating research proposals, grant proposals, and ultimately transforming that work into an Honors Thesis. I would like to thank Dr. Rachel Williams for her support throughout the entire process, as well as her knowledge in bringing all of my work together in creating this thesis. I would like to thank The Undergraduate Research Office and the National Institutes of Health for the procedural and financial support of this thesis.

Citations

1. Albini A, Morini M, D'Agostini F, Ferrari N, Campelli F et al (2001) Inhibition of angiogenesis- driven Kaposi's sarcoma tumor growth in nude mice by oral N-acetylcysteine. Cancer Res 61:8171–8178
2. Atalay M, Gordillo G, Roy S et al. (2003) Anti-angiogenic property of edible berry in a model of hemangioma. FEBS Lett 5(1-3): 252-257
3. Austyn, J.M. and Gordon, S. (1981) F4/80, a monoclonal antibody directed specifically against the mouse macrophage. Eur J Immunol 11: 805-815
4. Bagchi D, Roy S, Patel V et. al (2006) Safety and whole-body antioxidant potential of a novel anthocyanin-rich formulation of edible berries. Mol Cell Biochem. 1(1-2): 197-209
5. Fernandez Y, Bernabeu-Wittel M, Garcia-Morillo JS. (2009) Kaposiform hemangioendothelioma. European Journal of Internal Medicine 20:106–113
6. Golpon HA, Fadok VA, Taraseviciene-Stewart L, et al. (2004) Life after corpse engulfment: phagocytosis of apoptotic cells leads to VEGF secretion and cell growth. FASEB J. 18:1716-1718.
7. Gordillo, G, Fang, H, Khanna, S, Harper, J, Phillips, G, and Sen, C. (2009) Oral Administration of Blueberry Inhibits Angiogenic Tumor Growth and Enhances Survival of Mice with Endothelial Cell Neoplasm. Antioxidants & Redox Signaling 11: 47-58.
8. Gordillo, G, and Sen, C. (2009) Endothelial Cell Tumor Prevention with Berry Extracts: Clinical Problems, Molecular Mechanisms and Therapeutic Opportunities. Antioxidants & Redox Signaling 11: 117-130
9. Gordillo GM, Atalay M, Roy S, Sen CK (2002) Hemangioma model for in vivo angiogenesis: inducible oxidative stress and MCP-1 expression in EOMA cells. Meth Enzymol 352:422–432
10. Gordillo G, Fang H, Park HA, Roy S (2010) Nox-4 dependent nuclear H2O2 drives DNA oxida-tion resulting in 8-ohdg as urinary biomarker and hemangioendothelioma formation. AntioxidRedox Signal 12:10
11. Gordillo G, Onat D, Stockinger M, Roy S, Atalay M et al (2004) A key angiogenic role of monocyte chemoattractant protein-1 in hemangioendothelioma proliferation. Am J Physiol (Cell) 287:C866–C873
12. Haytowitz DB, Bhagwat S. (2010) USDA Database for ORAC of Selected Foods. 1: 1-18
13. Hoak JC, Warner ED, Cheng HF, Fry GL, Hankenson RR (1971) Hemangioma with thrombocytopenia and microangiopathic anemia (Kasabach-Merritt syndrome): an animal model. J Lab Clin Med 77:941–950
14. Islam MS, Protic O, Ciavattini A, Giannubilo SR, Tranquilli AL, Catherino WH, Castellucci M, Ciarmela P. (2014) Tranilast, an orally active antiallergic compound, inhibits extracellular matrix production in human uterine leiomyoma and myometrial cells. Fertil Steril 102(2): 597-606
15. Kakkad SM, Penet MF, Akhbardeh A, Pathak AP, Solaiyappan M, Raman V, Leibfritz D, Glunde K, Bhujwalla ZM. (2013) Hypoxic tumor environments exhibit disrupted collagen I fibers and low macromolecular transport. PLos One 8(12): e81869

16. Ki CS, Lin TY, Korc M, Lin CC. (2014) Thiol-ene hydrogels as desmoplasia-mimetic matrices for modeling pancreatic cancer cell growth, invasion, and drug resistance. Biomaterials 35(36): 9668-9677
17. Lannutti B, Gately S, Quevedo M, Soff G, Paller A (1997) Human angiostatin inhibits murine hemangioendothelioma tumor growth in vivo. Cancer Res 57:5277–5280
18. Lawson L.J et al. (1990) Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain. Neuroscience 39: 151-170
19. Martin T, Cardarelli PM, Parry GC, Felts KA, Cobb RR (1997) Cytokine induction of mono- cyte chemoattractant protein-1 gene expression in human endothelial cells depends on thecooperative action of NF-kappa B and AP-1. Eur J Immunol 27:1091–1097
20. Mills, Charles. M1 and M2 Macrophages: Oracles of Health and Disease (2012) 32:6 463-488
21. O'Reilly M, Brem H, Folkman J (1995) Treatment of murine hemangioendotheliomas with the angiogenesis inhibitor AGM-1470. J Pediatr Surg 30:325–330
22. Powers CJ, McLeskey SW, Wellstein A. (2000) Fibroblast growth factors, their receptors and signaling. Endocr Relat Cancer 7(3): 165-197
23. Reddy SM, Hsiao KH, Abernethy VE, et al. (2002) Phagocytosis of apoptotic cells by macrophages induces novel signaling events leading to cyto- kine-independent survival and inhibition of prolif- eration: activation of Akt and inhibition of extracel-lular signal-regulated kinases 1 and 2. J Immunol. 169:702-713
24. Roy S, Khanna S, Alessio HM, Vider J, Bagchi D et al (2002) Anti-angiogenic property of edible berries. Free Radic Res 36:1023–1031
25. Roy S, Khanna S, Sen CK (2008) Redox regulation of the VEGF signaling path and tissue vas- cularization: hydrogen peroxide, the common link between physical exercise and cutaneous wound healing. Free Radic Biol Med 44:180–192
26. Deshmane S, Kremlev S, Amini S et. al (2009) Monocyte Chemoattractant Protein-1 (MCP-1): An Overview. J Interferon Cytokine Res. 29(6): 313-326
27. Scholzen T, Gerdes J. (2000) The Ki-67 protein: from the known and the unknown. J Cell Physiol 182(3):311-22.
28. Sen CK, Packer L (1996) Antioxidant and redox regulation of gene transcription. FASEB J 10:709– 720
29. Sodagari HR, Farzaei MH, Bahramsoltani R et al. (2015). Dietary anthocyanins as a complementary medicinal approach for management of inflammatory bowel disease. Expert Rev Gastroenterol Hepatol 13:1-14
30. Strutz F, Okada H, Lo CW, Danoff T, Carone RL, Tomaszewski JE, Neilson EG. (1995) Identification and characterization of a fibroblast marker: FSP1. J Cell Biol 130(2): 393-405
31. Taraboletti G, Garofalo A, Belotti D, Drudis T, Borsotti P et al (1995) Inhibition of angio- genesis and murine hemangioma growth by batimastat, a synthetic inhibitor of matrix metalloproteinases. J Natl Cancer Inst 87:293–298
32. Wang C, Quevedo ME, Lannutti BJ, Gordon KB, Guo D et al (1999) In vivo gene therapy with interleukin-12 inhibits primary vascular tumor growth and induces apoptosis in a mouse model. J Invest Dermatol 112:775–781
33. Weigert, Andreas, Johann AM, Von Knethen A, et al. (2006) Apoptotic cells promote macrophage survival by releasing the antiapoptotic mediator sphingosine-1-

phosphate. Blood 108:5 1635-1642